Institute Report No. 293

Mutagenic Potential of DIGL-RP Solid Propellant in the Ames *Salmonella*/Mammalian Microsome Mutagenicity Test

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GENETIC TOXICOLOGY BRANCH
DIVISION OF TOXICOLOGY

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LETTERMAN ARMY INSTITUTE OF RESEARCH
PRESIDIO OF SAN FRANCISCO, CALIFORNIA 94129

Mutagenic Potential of DIGL-RP Solid Propellant in the Ames *Salmonella*/Mammalian Microsome Mutagenicity Test (Toxicology Series 150)--Sano and Korte

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Richard A. Kishimoto 28 Sept 88

for Edwin S. Beatrice (date)
COL, MC
Commanding

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The mutagenic potential of DIGL-RP solid propellant was assessed by using the Ames Salmonella/Mammalian Microsome Mutagenicity Test. Tester strains TA97, TA98, TA100, and TA102 were exposed to doses ranging from 5 mg/plate to 0.0016 mg/plate. The test compound was not mutagenic under conditions of this test.

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ABSTRACT

The mutagenic potential of DIGL-RP solid propellant was assessed by using the Ames *Salmonella*/Mammalian Microsome Mutagenicity Test. Tester strains TA97, TA98, TA100, and TA102 were exposed to doses ranging from 5 mg/plate to 0.0016 mg/plate. The test compound was not mutagenic under conditions of this test.

Key Words: Mutagenicity, Genetic Toxicology, Ames Test, DIGL-RP, DEGDN, Solid Propellant

PREFACE

TYPE REPORT: Ames Test GLP Study Report

TESTING FACILITY:

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Letterman Army Institute of Research
Presidio of San Francisco, CA 94129-6800

SPONSOR:

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US Army Biomedical Research and Development Laboratory
Fort Detrick, Frederick, MD 21701-5012
Project Officer: Gunda Reddy, PhD

PROJECT/WORK UNIT/APC: #3E162720A835/180/TLB0

GLP STUDY NUMBER: 85014

STUDY DIRECTOR: MAJ Don W. Korte Jr., PhD, MSC

PRINCIPAL INVESTIGATOR: SGT Steven K. Sano, BA

REPORT AND DATA MANAGEMENT: A copy of the final report, study protocol, retired stability and purity data on the test compound, tissues, and an aliquot of the test compound will be retained in the LAIR Archives.

TEST SUBSTANCE: DIGL-RP Solid Propellant

INCLUSIVE STUDY DATES: 19 Aug - 14 Sep 85

OBJECTIVE: The objective of this study was to determine the mutagenic potential of DIGL-RP solid propellant (LAIR Code TP057) by using the Ames *Salmonella*/Mammalian Microsome Mutagenicity Test.

ACKNOWLEDGMENTS

CPT John W. Harbell, PhD, MSC; SGT Lillie D. Witcher, BS; SP4 John R.G. Ryabik, BS; Mr. John Dacey; and Ms. Joanne Wong provided research assistance.

SIGNATURES OF PRINCIPAL SCIENTISTS INVOLVED IN THE STUDY

We, the undersigned, declare that GLP study number 85014 was performed under our supervision, according to the procedures described herein, and that this report is an accurate record of the results obtained.

Don W. Korte Jr 14 July 88

DON W. KORTE JR, PhD / DATE
MAJ, MSC
Study Director

Conrad Wheeler 14 July 88

CONRAD WHEELER, PhD/ DATE
DAC
Analytical chemist

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DEPARTMENT OF THE ARMY

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REPLY TO
ATTENTION OF:

SGRD-ULZ-QA (7Ø-1n) 15 September 1988

MEMORANDUM FOR RECORD

SUBJECT: GLP Compliance for GLP Study 85Ø14

1. This is to certify that in relation to LAIR GLP Study 85Ø14, the following inspections were made:

16 August 1985	-	Protocol Review
11 September 1985	-	Plate Incorporation
13 September 1985	-	Plate Counting

2. The institute report entitled "Mutagenic Potential of DIGL-RP Solid Propellant in the Ames Salmonella/Mammalian Microsome Mutagenicity Test," Toxicology Series 15Ø, was audited on 2Ø July 1988.

Carolyn M. Lewis
CAROLYN M. LEWIS
Chief, Quality Assurance

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Mutagenic Potential of DIGL-RP Solid Propellant in the Ames *Salmonella*/Mammalian Microsome Mutagenicity Test--Sano and Korte

INTRODUCTION

The Department of Defense is considering the use of diethyleneglycol dinitrate (DEGDN), triethyleneglycol dinitrate (TEGDN), or trimethylolethane trinitrate (TMETN) as a replacement for nitroglycerin in munition formulations. A "health effects" review conducted for the US Army Biomedical Research and Development Laboratory (USABRDL) identified numerous gaps in the toxicology database of these compounds (1). Consequently, USABRDL has tasked the Division of Toxicology, LAIR, to conduct an initial evaluation of the health effects of DEGDN, TMETN, TEGDN, and two DEGDN-based propellants, JA-2 and DIGL-RP. This initial evaluation includes the Ames mutagenicity test, acute oral toxicity tests in rats and mice, acute dermal toxicity tests in rabbits, dermal and ocular irritation studies in rabbits, and dermal sensitization studies in guinea pigs. This report contains the results of a study that assessed the mutagenic potential of DIGL-RP in the Ames *Salmonella*/Mammalian Microsome Mutagenicity Test.

The Ames *Salmonella*/Mammalian Microsome Mutagenicity Test is a short-term screening test that utilizes histidine auxotrophic mutant strains of *Salmonella typhimurium* to detect compounds that are potentially mutagenic in mammals. A mammalian microsomal enzyme system is incorporated in the test to increase sensitivity by simulating *in vivo* metabolic activation of the test compound. The Ames test is an inexpensive yet highly predictive and reliable test for detecting mutagenic activity and thus carcinogenic potential (2).

This evaluation of DIGL-RP utilizes a revision of the Ames *Salmonella*/Mammalian Microsome Mutagenicity Test (3). Two new tester strains, a frame-shift strain (TA97) and a strain carrying an ochre mutation on a multicopy plasmid (TA102), are added to the standard tester set. TA97 replaces TA1537, TA1535 and TA1538 which are removed from the recommended set. TA98 and TA100 are retained.

Objective of the Study

The objective of this study was to determine the mutagenic potential of DIGL-RP solid propellant (LAIR Code TP057) by using the revised Ames *Salmonella*/Mammalian Microsome Mutagenicity Test.

MATERIALS AND METHODS

Test Compound

Compound name: DIGL-RP Solid Propellant

Code number: LAIR Code No. TP057

Physical state: Solid

Source: Hercules Incorporated
Wilmington, Delaware

Storage: DIGL-RP was received from Radford Army Ammunition Plant (Radford, VA) and assigned the LAIR Code number TP057. The test compound was stored at room temperature (21°C) until used.

Chemical Properties/Analysis: Data provided by Hercules Inc., characterizing the chemical composition and purity of the test material, are presented in Appendix A along with confirmatory analysis of the test material performed by the Division of Toxicology, LAIR (Presidio of San Francisco, CA).

Test Solvent

The positive control chemicals and the test compound were dissolved in grade I dimethyl sulfoxide (lot 113F-0450) obtained from Sigma Chemical Co. (St. Louis, MO).

Chemical Preparation

DIGL-RP was stored at room temperature (21°C) until used. The solid propellant was ground into a fine powder with a liquid nitrogen freezer/mill Model # 6700 (Spex Industries). On the day of dosing, 300 mg of the test compound was measured into a sterile vial and dissolved in 6 ml of grade I dimethyl sulfoxide to achieve a 5% (w/v) solution. Aliquots of this solution were used to dose the test plates.

Test Strains

Salmonella strains TA97, TA98, TA100, and TA102, obtained directly from Dr. Bruce Ames, University of California, Berkeley, were used. These strains were maintained in our laboratory at -80°C. Quality control tests were run concurrently with the test substance to establish the validity of their special features and to determine the spontaneous reversion rate. Descriptions of the strains, their genetic markers, and the methods for strain validation are given in the LAIR SOP, OP-STX-1 (4).

Test Format

DIGL-RP was evaluated for mutagenic potential according to a revised Ames method (3). A detailed description of the methodology is given in LAIR SOP, OP-STX-1 (4).

Toxicity Tests

Toxicity tests were conducted to determine a sublethal concentration of the test substance. This toxicity level was found by using minimal glucose agar (MGA) plates, concentrations of DIGL-RP ranging from 1.6×10^{-3} mg/plate to 5 mg/plate, and approximately 10^8 cells of TA100 per plate. Top agar containing trace amounts of histidine and biotin was placed on the plates. Strain verification was confirmed on the bacteria, along with a determination of the spontaneous reversion rate. After incubation, the growth on the plates was observed. Since none of the plates showed a decrease in the number of macrocolonies (below the number in the spontaneous reversion plates) or an observable reduction in the density of the background lawn, a maximum "limit" dose of 5 mg/plate was used in the mutagenicity test.

Mutagenicity Test

The test substance was evaluated over a 1000-fold range of concentrations, decreasing from the minimum toxic level (the maximum or limit dose) by a dilution factor of 5, both with and without 0.5 ml of the S-9 microsome fraction. The S-9 was purchased from Microbiological Associates Inc. (Bethesda, MD). The optimal titer of this S-9, as determined by Microbiological Associates Inc., was 0.75 mg protein/plate. After all the ingredients were added, the top agar was mixed, then overlaid on MGA plates. These plates contained 2% glucose and Vogel Bonner "E" Concentrate (5). The water used in this medium and in all reagents came from a Technic Model 301 Reverse Osmosis Pre-Treatment Water System (Seattle, WA), LAIR SOP, OP-STX-94 (6). Plates were

incubated upside down in the dark at 37°C for 72 hr. Plates were prepared in triplicate and the average revertant counts were recorded. The average number of revertants at each dose level was compared to the average number of spontaneous revertants (negative control). The spontaneous reversion rate (with and without S-9) was monitored by averaging the counts from two determinations run simultaneously with the test compound. The spontaneous reversion rate was determined by inoculating one set of plates before and one set after the test compound plates so that any change in spontaneous reversion rate during the dosing procedure would be detected. This spontaneous reversion rate was also compared with historical values for this laboratory and those cited in Maron and Ames (3). Sterility and strain verification controls were run concurrently. All reagents, test compounds, and media were checked for sterility by plating samples of each on MGA media and incubating them at 37°C with the test plates. The *Salmonella* strains were verified by a standard battery of tests. The integrity of the different *Salmonella* strains used in the assay was verified by the following standard tests:

-Lack of growth (inhibition) in the presence of crystal violet which indicates that the prerequisite alteration of the lipopolysaccharide layer of the cell wall is present.

-Growth in the presence of ampicillin-impregnated disks which indicates the presence of an ampicillin-resistant R Factor.

-Lack of growth (inhibition) following exposure to ultraviolet light which indicates the absence of the DNA excision-repair mechanism (for all strains except TA102).

Four known mutagens were tested as positive controls to confirm the responsiveness of the strains to the mutation process. Each strain must be tested with at least one positive control but may be tested with several. These compounds, benzo[a]pyrene, 2-aminofluorene, 2-aminoanthracene and 4-nitroquinoline-n-oxide, were obtained from Sigma Chemical Co. (St. Louis, MO). The test compound and mutagens were handled during this study in accordance with the standards published in NIH Guidelines for the Laboratory Use of Chemical Carcinogens (DHHS Publication No. (NIH) 81-2385, May 1981).

Data Interpretation

According to Brusick (7), a compound is considered mutagenic if a positive dose response (correlated dose response) over three dose concentrations is achieved with at least the highest dose yielding a revertant colony count greater than or equal to twice the spontaneous colony count for the tester strains TA98 and TA100. A strong correlated dose response in strain TA100 without a doubling of the individual colony count may also be considered positive.

Maron and Ames (3) consider a compound mutagenic in tester strains TA97 and TA102 if a correlated dose response over three concentrations is achieved with the highest dose yielding a revertant colony count greater than or equal to twice the spontaneous colony count.

Deviations/Changes

A 72-hr rather than a 48-hr incubation period was used. According to Maron (personal communication, 1985), the additional 24-hr growth enables all of the revertant colonies, especially TA102, to be detected with the colony counter.

Storage of Raw Data and Final Report

A copy of the final report, study protocols, raw data, SOPs, and an aliquot of the test compound will be retained in the LAIR archives.

RESULTS

On 23 August 1985, the toxicity of DIGL-RP was determined (Table 1). For this experiment all sterility, strain verification, and negative controls were normal (Table 1). No toxicity was observed after exposure of the tester strain (TA100) to the highest dose used (5 mg/plate).

Normal results were obtained for all sterility and strain verification tests during the Ames Test performed on 27-30 August 1985 (Table 2). DIGL-RP did not induce any appreciable increase in the revertant colony counts relative to those of the negative control cultures (Table 3).

A copy of the raw data is included in Appendix B.

TABLE 1: TOXICITY DETERMINATION FOR DIGL-RP

GLP STUDY NUMBER 85014 23 Aug 1985 PERFORMED BY SANO/WONG

TOXICITY DETERMINATION REVERTANT PLATE COUNT (TA100)

CONCENTRATION OF TEST COMPOUND	MEAN	(1SD)	BACKGROUND LAWN*
START RUN NEGATIVE CONTROL	102	(14.0)	NL
5.0 mg/plate	67	(6.6)	NL
1.0 mg/plate	71	(7.2)	NL
0.2 mg/plate	83	(11.1)	NL
0.04 mg/plate	77	(10.1)	NL
0.008 mg/plate	86	(1.2)	NL
0.0016 mg/plate	91	(8.3)	NL
END RUN NEGATIVE CONTROL	96	(8.1)	NL

STRAIN VERIFICATION FOR TOXICITY DETERMINATION (TA100)

HISTIDINE REQUIREMENT	NG*	
AMPICILLIN RESISTANCE	G	
UV	NG	
CRYSTAL VIOLET SENSITIVITY (ZONE SIZE)	NG	(12mm)
STERILITY CONTROL	NG	

STERILITY CONTROL FOR TOXICITY DETERMINATION

MATERIAL TESTED	OBSERVATION*
MINIMAL GLUCOSE AGAR PLATES	NG
TOP AGAR	NG
DILUENT WATER	NG
NUTRIENT BROTH	NG
TEST COMPOUND (HIGHEST DOSE)	NG

* NL = Normal Lawn G = Growth NG = No Growth

TABLE 2: STRAIN VERIFICATION AND STERILITY TESTING FOR THE MUTAGENICITY DETERMINATION OF DIGL-RP (TP057)

GLP STUDY NUMBER 85014 12 SEP 1985 PERFORMED BY SANO/WONG

STRAIN VERIFICATION

	OBSERVATIONS*			
STRAINS	TA97	TA98	TA 100	TA102
HISTIDINE REQUIREMENT	NG	NG	NG	NG
AMPICILLIN RESISTANCE	G	G	G	G
UV REPAIR	NG	NG	NG	G
CRYSTAL VIOLET				
SENSITIVITY	NG	NG	NG	NG
(ZONE SIZE)	(12mm)	(10mm)	(12mm)	(12mm)
STERILITY CONTROL	NG	NG	NG	NG

STERILITY CONTROL FOR MUTAGENICITY DETERMINATION

MATERIAL TESTED	OBSERVATION*
MINIMAL GLUCOSE AGAR PLATES	NG
TOP AGAR	NG
DILUENT WATER	NG
NUTRIENT BROTH	NG
TEST COMPOUND (HIGHEST DOSE)	NG

* NL = Normal Lawn G = Growth NG = No Growth

TABLE 3: MUTAGENICITY ASSAY FOR DIGL-RP (TP057)†

STUDY NUMBER: 85014 DATE: 12 SEPT 85 PERFORMED BY SANO/WONG

COMPOUND	DOSE	TA97	TA98	TA100	TA102
		WITHOUT S-9			
NEG CONTROL	0.0 mg/ml	61 (7.4)	28 (1.9)	106 (14.7)	153 (19.0)
NQNO*	2.0 µg/ml	72 (6.7)	278 (28.0)	1229 (69.3)	1189 (94.3)
TP057	5.0 mg/plate	67 (6.1)	24 (5.6)	83 (4.6)	125 (4.5)
TP057	1.0 mg/plate	64 (5.0)	19 (1.0)	90 (6.1)	119 (9.6)
TP057	0.2 mg/plate	60 (0.6)	21 (3.0)	83 (7.0)	111 (8.3)
TP057	0.04 mg/plate	74 (5.1)	25 (7.0)	70 (11.9)	109 (19.9)
TP057	0.008 mg/plate	56 (3.8)	22 (4.9)	80 (1.5)	130 (13.0)
TP057	0.0016 mg/plate	51 (8.0)	24 (9.7)	89 (6.1)	143 (9.5)
		WITH S-9			
NEG CONTROL	0.0 mg/ml	65 (3.9)	27 (6.0)	94 (14.3)	173 (51.5)
AF*	2.0 µg/ml	172 (4.0)	1654 (44.3)	1118 (74.5)	289 (31.4)
BP*	2.0 µg/ml		412 (103.5)	523 (10.4)	
AA*	2.0 µg/ml		1754 (89.7)	1792 (88.6)	
TP057	5.0 mg/plate	66 (4.6)	26 (4.4)	97 (4.0)	210 (14.5)
TP057	1.0 mg/plate	62 (18.8)	22 (1.5)	81 (16.3)	196 (5.9)
TP057	0.2 mg/plate	62 (6.7)	24 (3.6)	89 (2.6)	191 (20.0)
TP057	0.04 mg/plate	63 (5.0)	22 (3.5)	90 (11.6)	156 (29.0)
TP057	0.008 mg/plate	60 (8.4)	25 (8.5)	84 (19.3)	164 (20.1)
TP057	0.0016 mg/plate	68 (4.2)	28 (4.7)	79 (19.2)	162 (13.9)

† Values represent the mean number of revertants/plate (± standard deviation)
* NQNO = 4-nitroquinoline-n-oxide, AF = 2-aminofluorene, BP = benzo[a]pyrene, AA = 2-aminoanthracene

DISCUSSION

Certain test criteria must be satisfied before an Ames test can be considered a valid assessment of a compound's mutagenic potential. First, the special features of the Ames strains must be verified. These features include demonstration of ampicillin resistance, alterations in the LP layer, and deficiency in DNA excision-repair (except TA102). Second, the *Salmonella* strains must be susceptible to mutation by known mutagens. Third, the optimal concentration of the test compound must be determined by treating TA100 with a broad range of doses and observing the potential toxic effects on formation of macrocolonies and microcolonies. If these tests are performed and expected data are obtained, then the results of the Ames test can be considered valid.

After validation of bacterial strains and selection of optimal sublethal doses, DIGL-RP was evaluated in the Ames test. Criteria for a positive response are a correlated dose-response relationship and a twofold increase in revertant colony counts relative to the respective negative control counts (3,4,7). DIGL-RP did not induce the requisite dose-response relationship or the increase in revertant colony counts necessary for a positive response. Thus, the results of this test indicate that DIGL-RP is not mutagenic when evaluated in the Ames test.

CONCLUSION

DIGL-RP was evaluated for mutagenic potential in the Ames Test, both in the presence and absence of metabolic activation, and did not induce a positive mutagenic response under conditions of this study.

REFERENCES

1. Holleman JW, Ross RH, Carroll JW. Problem definition study on the health effects of diethyleneglycol dinitrate, triethyleneglycol dinitrate, and trimethylolethane trinitrate and their respective combustion products. Frederick, Maryland: US Army Medical Bioengineering Research and Development Laboratory, 1983, DTIC No. ADA 127846.

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6. Operation of the Technic Model 301 Reverse Osmosis Pre-Treatment Water System and the Corning Model MP-1 Glass Still. LAIR Standard Operating Procedure OP-STX-94, Presidio of San Francisco, California: Letterman Army Institute of Research, 29 July 1985.

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Appendix A: CHEMICAL DATA

Name of Test Substance: DIGL-RP Solid Propellant

Composition/Analytical Data: See appended data sheet for information supplied by source

LAIR Code No.: TP057

Physical State: Solid Black Cylinders

Preparation of Test Substance for Dosing:

The cylinders of DIGL were ground to a fine powder under liquid nitrogen using a Spex freezer mill. After grinding, the powder was seived through an 80-mesh screen and dissolved in DMSO.

Source: Radford Army Ammunition Plant, Radford, Virginia (prime contractor: Hercules Inc., Wilmington, Delaware).

Lot No.: RAD83M001S169

Appendix A (cont.): CHEMICAL DATA

DATA SHEET

FORMULA

DIGL-RP

Ingredient	Finished Propellant Percentage
Nitrocellulose 13.10% ± 0.05% Nitrogen 6-12 seconds viscosity	62.50 ± 2.00
Diethyleneglycol Dinitrate (DEGDN)	36.70 ± 1.50
Ethyl Centralite (EC)	0.25 ± 0.05
Akardit II	0.25 0.45 ± 0.20
Magnesium Oxide	0.05 Max
Graphite (Chg 5)	0.05 Max
TOTAL:	100.00

Appendix B: INDIVIDUAL PLATE SCORES

TOXICITY DETERMINATION WITH TA100

COMPOUND	DOSE/plate	PLATE 1	PLATE 2	PLATE 3
NEGATIVE CONTROL (Start Run)		116	88	102
TP057	5.0 mg	60	68	73
TP057	1.0 mg	69	79	65
TP057	0.2 mg	71	85	93
TP057	0.04 mg	76	68	88
TP057	0.008 mg	87	85	85
TP057	0.0016 mg	94	98	82
NEGATIVE CONTROL (End Run)		101	87	101

Appendix B (cont.): INDIVIDUAL PLATE SCORES

MUTAGENICITY TESTS WITHOUT S-9

COMPOUND	DOSE/plate	TA97	TA98	TA100	TA102
NEG CONTROL (start run)		70	27	93	150
		53	30	100	128
		61	30	111	158
NEG CONTROL (END RUN)		53	29	130	145
		59	25	113	153
		69	28	91	186
NQNO*	2.0 µg	76	257	1156	1294
		75	268	1237	1111
		64	310	1294	1163
TP057	5.0 mg	70	18	84	130
		71	25	78	125
		60	29	87	121
TP057	1.0 mg	59	20	95	123
		63	18	91	108
		69	19	83	126
TP057	0.2 mg	59	24	80	120
		60	21	78	108
		60	18	91	104
TP057	0.04 mg	80	17	83	98
		70	30	66	132
		73	28	60	97
TP057	0.008 mg	54	24	81	122
		53	16	78	123
		60	25	80	145
TP057	0.0016 mg	43	13	94	153
		50	26	82	134
		59	32	90	143

* 4-nitroquinoline-n-oxide

Appendix B (cont.): INDIVIDUAL PLATE SCORES

MUTAGENICITY TESTS WITH S-9					
COMPOUND	DOSE/plate	TA97	TA98	TA100	TA102
NEG CONTROL		66	21	90	213
(Start Run)		59	24	122	221
		70	25	91	224
NEG CONTROL		63	36	88	121
(End Run)		64	33	81	126
		68	23	91	130
2-aminofluorene	2.0 µg	168	1702	1035	264
		173	1644	1140	278
		176	1615	1179	324
benzo[a]pyrene	2.0 µg		309	526	
			412	531	
			516	511	
2-aminoanthracene	2.0 µg		1847	1819	
			1746	1693	
			1668	1864	
TP057	5.0 mg	63	31	93	210
		63	24	96	196
		71	23	101	225
TP057	1.0 mg	72	21	87	198
		73	22	63	200
		40	24	94	189
TP057	0.2 mg	70	20	90	208
		59	27	91	196
		58	25	86	169
TP057	0.04 mg	68	24	78	185
		63	24	101	156
		58	18	92	127
TP057	0.008 mg	65	24	96	187
		64	34	62	155
		50	17	95	150
TP057	0.0016 mg	63	30	58	174
		69	32	96	166
		71	23	82	147

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